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Author: Katarzyna Hupert-Kocurek, Urszula Guzik, Danuta Wojcieszyńska

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Characterization of catechol 2,3-dioxygenase from *Planococcus* sp. strain S5 induced by high phenol concentration#

Katarzyna Hupert-Kocurek, Urszula Guzik✉ and Danuta Wojcieszyńska

Department of Biochemistry, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Katowice, Poland

This study aimed at characterization of a new catechol 2,3-dioxygenase isolated from a Gram-positive bacterium able to utilize phenol as the sole carbon and energy source. *Planococcus* sp. strain S5 grown on 1 or 2 mM phenol showed activity of both a catechol 1,2- and catechol 2,3-dioxygenase while at a higher concentrations of phenol only catechol 2,3-dioxygenase activity was observed. The enzyme was optimally active at 60°C and pH 8.0. Kinetic studies showed that the K_m and V_{max} of the enzyme were 42.70 μM and 329.96 mU, respectively. The catechol 2,3-dioxygenase showed the following relative *meta*-cleavage activities for various catechols tested: catechol (100%), 3-methylcatechol (13.67%), 4-methylcatechol (106.33%) and 4-chlorocatechol (203.80%). The high reactivity of this enzyme towards 4-chlorocatechol is different from that observed for other catechol 2,3-dioxygenases. Nucleotide sequencing and homology search revealed that the gene encoding the S5 catechol 2,3-dioxygenase shared the greatest homology with the known genes encoding isoenzymes from Gram-negative *Pseudomonas* strains.

Keywords: catechol 2,3- dioxygenase, phenol degradation, *Planococcus*

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INTRODUCTION

Many bacteria and other microorganisms can grow on various aromatics like benzene, naphthalene, phenol and its derivatives, as the sole source of carbon and energy. Under aerobic conditions biodegradation of aromatic compounds involves their conversion into dihydroxylated intermediates (e.g., catechol or its alkyl- or chloro-substituted derivatives) which are then further metabolized by intradiol or extradiol dioxygenases (Bugg, 2003; Palaniandavar & Mayilmurugan, 2007). These ring-cleaving enzymes catalyze the addition of both atoms of molecular oxygen into the aromatic ring (Okuta *et al.*, 1998). The intradiol enzymes incorporate the two oxygen atoms between the vicinal hydroxyl groups, while the extradiol dioxygenases cleave the aromatic ring of the substrate outside the two hydroxyl groups and produce muconic semialdehyde (Viggiani *et al.*, 2004; Kalogeris *et al.*, 2006). Under aerobic conditions microbial biodegradation of phenol can occur either through the *ortho* or the *meta* pathway (Harayama & Rekik 1989; Bugg, 2003; Fortin *et al.*, 2005; Bugg & Ramaswamy, 2008).

Due to their key role in the degradation of aromatic ring, intradiol and extradiol dioxygenases have been extensively studied in the last decades. Catechol 2,3-dioxygenases (C23Os) belong to the extradiol dioxygenases family. To data, they have been found in numerous Gram-negative (*Pseudomonas*, *Sphingomonas, Acinetobacter, Ralstonia, Burkholderia, Stenotrophomonas*) and Gram-positive (*Nocardia*, *Rhodococcus* and *Bacillus*) strains (Müller *et al.*, 1996; Milo *et al.*, 1999; Sei *et al.*, 1999; Jiang *et al.,* 2004; Junca *et al.,* 2004; Li *et al.*, 2004; Viggiani *et al.,* 2004; Kim *et al.*, 2005; Kasuga *et al.,* 2007; Ma *et al.,* 2010; Wei *et al.,* 2010; Tancsics *et al.,* 2010; Wojcieszyńska *et al.,* 2011). All known catechol 2,3-dioxygenases are homotetramers. Most of them contain non-heme iron Fe (II) in their active site but some are also active with Mn (II) (Sato *et al.*, 1997; Bugg, 2003; Hatta *et al.*, 2003; Viggiani *et al.*, 2004; Vaillancourt *et al.*, 2006).

Planococcus sp. strain S5, a Gram-positive bacterium isolated from activated sludge from a sewage treatment plant in Bytom Miechowice, Poland, is able to grow on salicylate or benzoate (Łabużek *et al.*, 2003). As we previously reported, the S5 strain grown on salicylate expressed both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities, while when grown on benzoate it only expressed catechol 1,2-dioxygenase activity. To date, only two *Planococcus* strains have been reported to degrade aromatic compounds: *Planococcus* sp. strain ZD22 and *Planococcus* sp. (Li *et al.,* 2006; Dalal *et al.,* 2012). In 2001, Engelhardt *et al.* described *Planococcus alkanoclasticus* sp. nov. isolated from intertidal beach sediment capable of degrading linear alkanes but not aromatic hydrocarbons. Most of the *Planococcus* bacteria are known to be moderately halophilic (Romano *et al.,* 2003; Sprott *et al.,* 2003; Li *et al.,* 2006). Moreover, some of these strains show heavy metal resistance (Chowdhury *et al.,* 2003; Nithya *et al.,* 2011) and an ability to produce surfactants (Jacobucci *et al.,* 2009). Because of the above properties, bacteria from the genus *Planococcus* could be useful in bioremediation of extreme contaminated environments.

In this study we present the ability of *Planococcus* strain S5 to degrade phenol by using catechol 1,2-dioxygenase and catechol 2,3-dioxygenase or only catechol 2,3-dioxygenase depending on the initial aromatic substrate concentration. The main aim of our work was to identify and characterize the catechol 2,3-dioxygenase of strain S5. The high reactivity of this enzyme towards 4-chlorocatechol distinguishes this protein from previously characterized catechol 2,3-dioxygenases. The nucleotide sequence of the isolated gene for C23O from the *Plano-*

✉e-mail: urszula.guzik@us.edu.pl
#GenBank ID: HQ223337
Abbreviations: C23O, catechol 2,3-dioxygenases; C12O, catechol 1,2-dioxygenases; CCMA, *cis,cis*-muconic acid; 2-HMS, 2-hydroxymuconic semialdehyde; K_m, Michaelis-Menten constant; MSM, mineral salts medium; V_{max}, maximum velocity

coccus strain was determined and compared with those of other known C23Os.

EXPERIMENTAL PROCEDURES

Media and culture conditions. *Planococcus* sp. strain S5, isolated from activated sludge of a sewage treatment plant in Bytom Miechowice, Poland (Łabużek *et al.*, 2003), was enriched in mineral salts medium (MSM) containing (g L^{-1}): $Na_2HPO_4 \cdot 12H_2O$ 3.78, KH_2PO_4 0.5, NH_4Cl 5, $MgSO_4 \cdot 7H_2O$ 0.2, 0.01 yeast extract. After autoclaving, phenol was added to the medium as the sole carbon source. Cultures were incubated at 30°C with agitation at 125 rpm for 24 hours. Analytical grade phenol was obtained from MERCK (Germany). All other chemicals used in this study were of the highest analytical grade available.

Phenol degradation by *Planococcus* sp. strain S5. For phenol degradation experiments, bacterial cultures in MSM were inoculated to the final optical density of about 0.1 at λ=600 nm into 100 mL of liquid MSM medium with phenol (1–4 mM) and then incubated as above for 9 hours. Culture samples were collected every hour and OD_{600nm} was measured. The concentration of phenol was determined with a colorimetric method using *p*-nitroaniline (Lurie & Rybnikova, 1986).

Enzyme assays. Cells in MSM liquid medium (with 1–4 mM phenol as the sole carbon source) were harvested in the late exponential growth phase and centrifuged at 4500 × *g* for 15 min at 4°C. Subsequently, the cells were washed with 50 mM phosphate buffer, pH 7.5, resuspended in the same buffer at an OD_{600} of 1.0 and disrupted by pulsed sonication (Vibra Cell TM) 6 times for 15 s each. The disrupted cell suspension was centrifuged at 9000 × *g* for 30 min at 4°C to remove cell debris. The clear supernatant was used as a crude extract for enzymes assays (Hegeman, 1966).

The activity of catechol 1,2-dioxygenase was determined spectrophotometrically at 260 nm by quantitating the formation of *cis,cis*-muconic acid (CCMA) (ε_{CCMA} = 16 800 M^{-1} cm^{-1}) at 40°C. The reaction mixture contained 20 μL of catechol (50 mM), 67 μL of Na_2EDTA (20 mM), 893 μL of phosphate buffer pH 7.5 (50 mM) and 20 μL of crude bacterial extract in a total volume of 1 mL (Hegeman, 1966). The crude enzyme extract was incubated with 5% H_2O_2 prior to the determination of catechol 1,2-dioxygenase activity to inactivate catechol 2,3-dioxygenase (Nakazawa & Yokota, 1973).

In order to determine catechol 2,3-dioxygenase activity, the formation of 2-hydroxymuconic semialdehyde (2-HMS) was measured at 375 nm ($\varepsilon_{2\text{-HMS}}$ = 36 000 M^{-1} cm^{-1}) in a reaction mixture containing 20 μL of catechol (50 mM), 960 μL of phosphate buffer pH 7.5 (50 mM) and 20 μL of crude extract in a total volume of 1 mL at 40°C (Feist & Hegeman, 1969). Control reactions (without crude extract) were performed for each assay. Protein concentrations of the crude extract was determined by the Bradford method (Bradford, 1976). One unit of C12O and C23O activity was defined as the enzyme amount generating 1 μmol of product per minute at 40°C. The specific activity is given in U per gram of protein.

Substrate specificity of catechol 2,3-dioxygenase. The substrate specificity of the catechol 2,3-dioxygenase was examined with 3-methylcatechol, 4-methylcatechol, and 4-chlorocatechol. The aromatic ring cleavage was determined spectrophotometrically by measuring the increase in the absorbance at the corresponding wavelength of each *meta*-cleavage product formed. The activity of C23O was assayed under the reaction conditions described above, using the aromatic compounds tested instead of catechol as a substrate. The molar extinction coefficient used for the product from 3-methylcatechol was 13 800 M^{-1} cm^{-1} (at 388 nm), from 4-methylcatechol 28 100 M^{-1} cm^{-1} (at 382 nm) (Bayly *et al.*, 1966), and from 4-chlorocatechol 40 000 M^{-1} cm^{-1} (at 379 nm) (Bae *et al.*, 1996).

pH and temperature optima of catechol 2,3-dioxygenase. The effect of pH on catechol 2,3-dioxygenase activity was determined by measuring the activity at 40°C over the pH range of 1.0–10.8 using the following buffers: 0.05 M glycine-HCl (pH 1.0 to 2.0), 0.05 M phosphate-citrate (pH 3.0 to 5.0), 0.05 M Sörensen's phosphate (pH 6.0 to 8.0) and 0.05 M borate (pH 9.0).

The optimum temperature was determined by assaying the enzyme activity at various temperatures (5–90°C) in 50 mM Tris/HCl buffer (pH 8.0). The enzyme and substrate solutions were pre-incubated, mixed and the enzymatic reaction was then carried out at the same temperature.

Determination of temperature stability. The thermal stability of the enzyme was determined by incubating the enzyme and enzymatic reaction mixtures at 40°C, 50°C and 60°C for 60 min and measuring the activity at 5, 10, 15 and 20 min of incubation at the same temperatures. Additionally, activity of the enzyme incubated at 40°C was measured after 30 and 60 min of incubation.

Determination of kinetic constants of catechol 2,3-dioxygenase. The catalytic parameters (Michaelis-Menten constant, K_m, and maximum velocity, V_{max}) were calculated by measuring the initial linear rates of the enzymatic reaction after the addition of different concentrations of catechol ranging from 0 to 200 μM at 40°C. Three independent measurements were carried out for each substrate concentration. K_m and V_{max} were calculated from Haldane iteration.

Identification and sequencing of catechol 2,3-dioxygenase gene. Plasmid and chromosomal DNA extraction, gel electrophoresis and PCR were performed by standard procedures (Sambrook *et al.*, 1989). The C23O gene was amplified using primers: 2,3D_zewF (ATGAAAAAAGGCGTAATGCGC) as forward and 2,3D_zewR (AGCACGGTCATGAAACGTTCGTTC) as the reverse primer designed based on the regions of catechol 2,3-dioxygenases with high sequence conservation allowing amplification of the entire gene (Wojcieszyńska *et al.*, 2011). Primers were purchased from IBB PAN (Warsaw, Poland). PCR amplification was carried out in a PTC-150 MiniCycler (MJ Research, USA). PCR reactions were performed in 25 μL volumes containing approximately 0.1 μg of template DNA (plasmid or chromosomal DNA), 0.5 μM of each primer, 1× *Pfu* buffer (MBI Fermentas), 3% (v/v) DMSO (Sigma), 0.2 mM of each dNTP, 1 U *Pfu* DNA polymerase (MBI Fermentas). PCR amplification was performed under the following conditions: initial denaturing 5 min at 94°C; 10 cycles of 1 min at 94°C, 30 s at 59°C, 1 min at 72°C; 10 cycles of 1 min at 94°C, 30 s at 57°C, 1 min at 72°C; 15 cycles of 1 min at 94°C, 30 s at 55°C 1 min at 72°C plus an additional 5 min elongation at 72°C. Aliquots (10 μL) of the PCR products were analyzed by electrophoresis on a 1.0% agarose gel stained with 0.5 μg mL^{-1} ethidium bromide. Gene sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem) and an AbiPrism_3100 Genetic Analyzer. Computer analysis and processing of sequence information were performed with the program Chromas LITE (Technely-

sium Pty, Tewantin, Australia). The obtained sequence of catechol 2,3-dioxygenase gene was compared with sequences available at the NCBI data base followed by multiple sequence alignment using the CLC FreeWorkbench software (CLC Bio A/S, Aarhus, Denmark). The nucleotide sequence of the catechol 2,3-dioxygenase gene from *Planococcus* sp. strain S5 has been deposited in the GenBank database of NCBI under the accession number HQ223337.

RESULTS AND DISCUSSION

Degradation of phenol by strain S5

Planococcus strain S5 was grown in MSM supplemented with 1–4 mM phenol. When S5 was grown with the initial phenol concentration of 1 mM or 2 mM, the phenol was degraded completely within 5 or 7 hours, respectively (Fig. 1A, B). At higher initial phenol concentrations in the medium (3–4 mM), the lag period of the culture increased in comparison to that growing at lower phenol concentrations, which could be connected with an inhibitory effect of high concentrations of phenol. However, the maximum growth of bacterial biomass was observed under high phenol concentrations (Fig. 1C, D).

Depending on the initial phenol concentration, an activity of both C12O and C23O or only C23O was observed in the crude cell extract. When cells were growing on 1 mM phenol, both enzymes were active but the activity of C23O was slightly lower (Table 1). In the presence of 2 mM phenol, both enzymes were induced and the activity of C23O was twice that of C12O (Table 1). Generally, degradation of phenol occurs either through the *ortho*- or *meta*-cleavage pathway (Dong *et al.*, 1992; Ng *et al.*, 1994; Powlowski & Shingler, 1994; Aneez Ahamad *et al.*, 1996; Duffner *et al.*, 2000). Simultaneous induction of both enzymes splitting the catechol ring was observed by Heinaru *et al.* (2000) in two out of 39 phenol- degrading strains, and by Djokić *et al.* (2011a, b) in two bacilli strains designated as PS1 and PS11. Activity of both enzymes was also determined in *Comamonas* strain PND-3 and *Cupriavidus* strain PND-6 (Dong *et al.*, 2008). However, in contrast to our results, the activity of C23O from the PND-3 and PND-6 strains was sig-

Table 1. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity in cell-free extracts of *Planococcus* sp. strain S5 grown on different initial phenol concentrations

Initial phenol concentration (mM)	Specific activity[a] (U g^{-1} protein)	
	Catechol 1,2-dioxygenase	Catechol 2,3-dioxygenase
1	16.6 ± 0.75	13.6 ± 0.69
2	10.8 ± 0.76	22.0 ± 0.60
3	0.00 ± 0.00	41.5 ± 0.58
4	0.00 ± 0.00	43.6 ± 0.66

[a]S5 strain was grown in 250 mL MSM containing 1–4 mM phenol to stationary phase before the enzyme activity determination. Data are expressed as mean ± standard deviation (n=3).

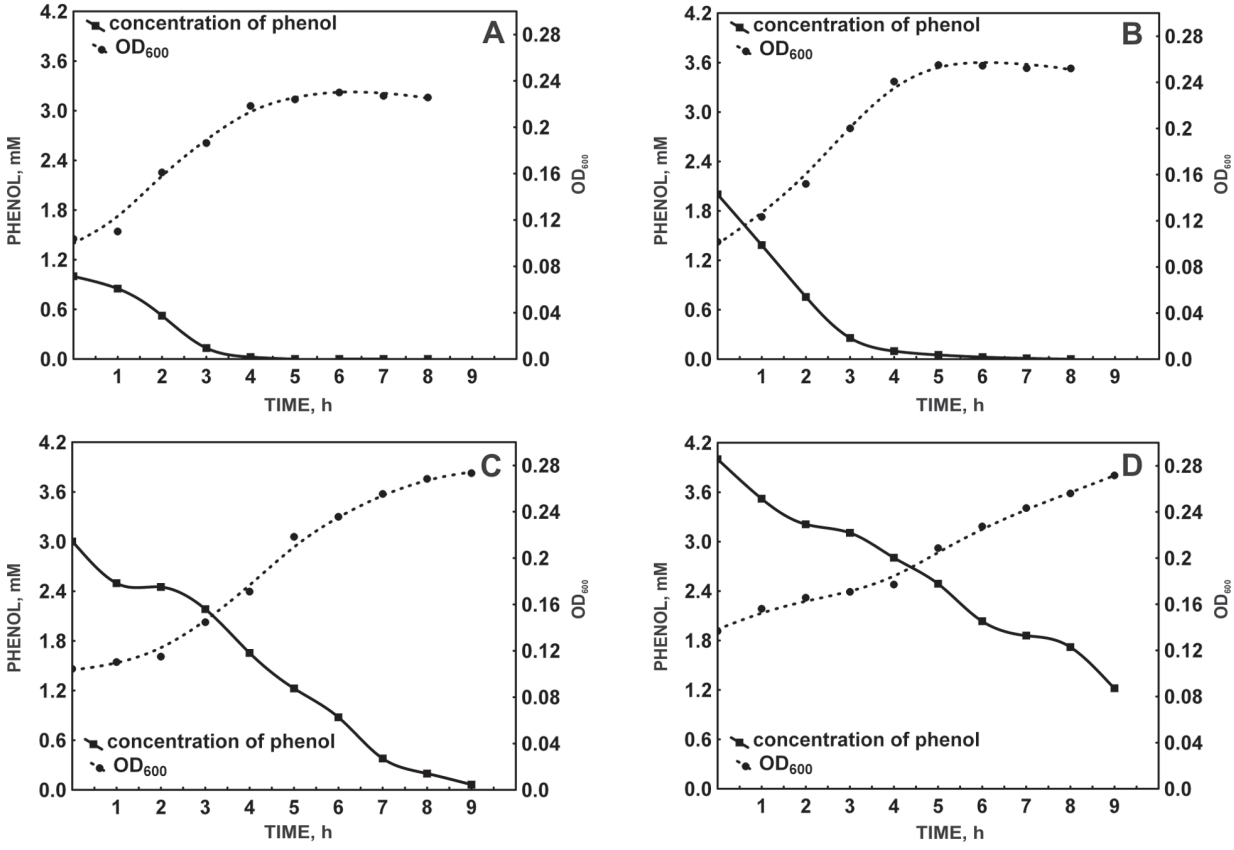

Figure 1. Time-course of phenol degradation and growth curve for *Planococcus sp.* S5 strain in media with different initial phenol concentration.
Liquid MSM contained initially 1 mM **(A)**, 2 mM **(B)**, 3 mM **(C)** or 4 mM **(D)** phenol. The results are average of triplicate independent experiments.

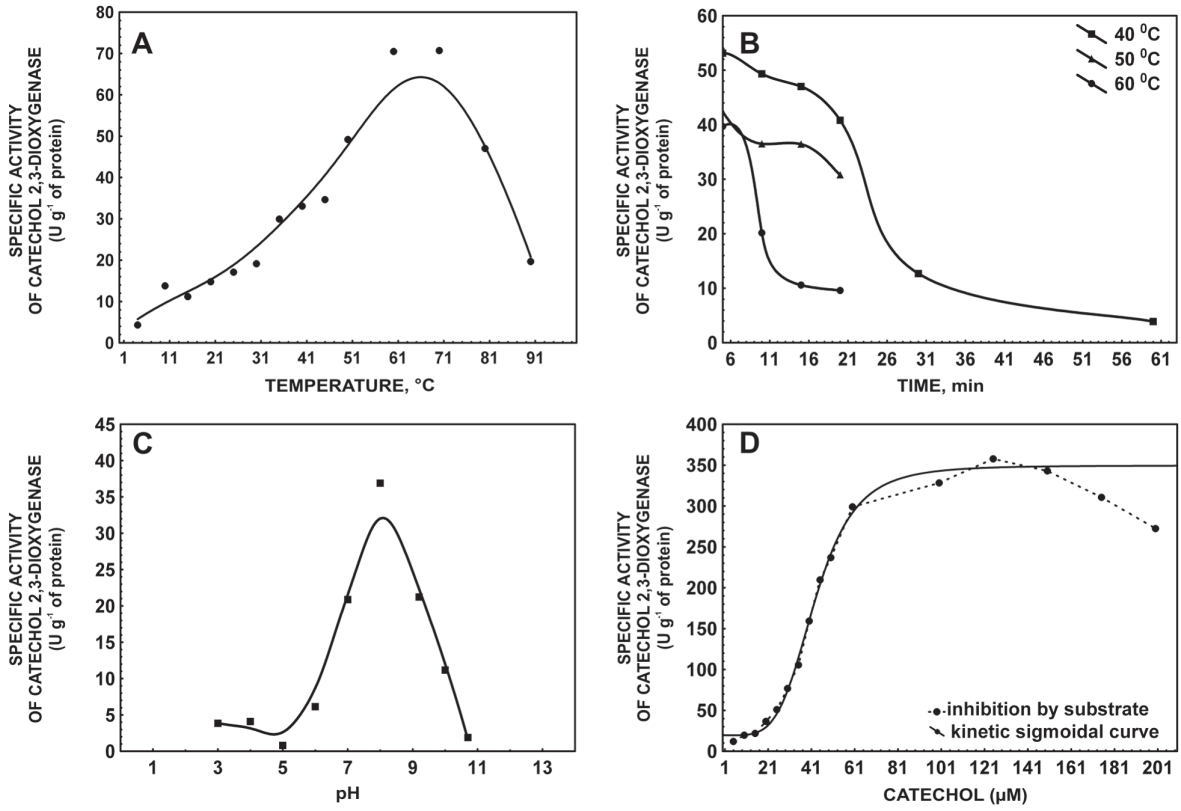

Figure 2. Effect of various factors on catechol 2,3-dioxygenase activity in *Planococcus sp.* S5 cell extract
(A) Ttemperature; **(B)** thermal stability**; (C)** pH; and **(D)** substrate concentration. Data points are averages of three independent experiments.

nificantly lower than that of C12O from those strains. At 3 or 4 mM initial phenol concentration in growth medium only the activity of C23O was observed in *Planococcus* crude extract (Table 1). It is generally known that C23O, the *meta*-pathway enzyme, is induced by the substrate while the *ortho*-cleavage pathway is induced by *cis, cis*-muconate, the product of the catechol ring fission (Williams *et al*., 1975; Song *et al*., 2000; Cao *et al*., 2008). When the S5 strain was grown in mineral medium with glucose, no activity of C23O and slight activity of C12O were observed (data not shown). As a result of the minimal activity of C12O, *cis, cis*-muconate was produced. Thus, at lower phenol concentrations both pathways could coexist in the same cell. Higher concentrations of phenol increase C23O expression, which results in the conversion of catechol into 2-hydroxymuconic semialdehyde and does not allow the accumulation of *cis, cis*-muconate. Consequently, this inhibits the induction of the *ortho*-pathway enzyme. Induction of the *meta*-pathway enzymes at higher concentrations of an aromatic substrate was also observed by Loh & Chua (2002) and Cao *et al.* (2008) during their studies on benzoate degradation by *Pseudomonas putida* ATCC49451. Benzoate is a known *ortho*-pathway inducer. It is speculated that induction of the *meta*-pathway at high concentrations of this substrate could be connected with accumulation of dihydroxybenzoate, a product of benzoate hydroxylation (Loh & Chua, 2002).

Catechol 2,3-dioxygenase activity and substrate specificity

Lower initial phenol concentrations induced both C12O and C23O activity (Table 1). In the presence of 4 mM phenol only the activity of C23O was observed (Table 1). Phenol at this concentration was used in studies on the enzymatic activity, substrate specificity and kinetic parameters of catechol 2,3-dioxygenase.

The aromatic ring-fission activity of C23O from *Planococcus* sp. S5 was tested at different temperatures and with buffers of various pH (see Materials and Methods). The effect of temperature on the enzyme activity was investigated in the range 5–90°C. Although the enzyme exhibited the highest activity at 60°C (Fig. 2A), it was unstable at that temperature. The half-life of the enzyme at 60°C was only 10 min (Fig. 2B). The enzyme has lost about 30% of its activity after 20 minutes at 50°C and its activity could still be detected even at 90°C (Fig. 2A). A slightly higher optimum temperature was observed for the C23O enzyme from *Bacillus thermoleovorans* A2 (Milo *et al*., 1999). Its half-life at 70°C was 1.5 min under aerobic condition and 10 min in a nitrogen atmosphere (Milo *et al*., 1999). C23Os isolated from *Pseudomonas* strains ZJF08 and S-47 were optimally active at 40°C and 30–35°C, respectively (Kim *et al*., 1997; Zou *et al*., 2007).

The optimal pH for the C23O from strain S5 was determined using catechol as a substrate. The enzyme showed the highest activity (38 U g^{-1} protein) at pH 8.0 (Fig. 2C) , which is similar to catechol 2,3-dioxygenase from *Pseudomonas* sp. AW-2 (Murakami *et al*., 1998). However, in contrast to C23O from strain S5, the dioxygenase from *Pseudomonas* sp. AW-2 was stable at pH 6.0–7.5. The optimal pH for C23O from the thermoacidophilic *Sulfolobus solfataricus* strain 98/2 was 7.0–7.5. The enzyme was still 65% active at pH 8.0, 61% active at pH 9.0 and 29% active at pH 4.0 (Murakami *et al*., 1998), while C23O from strain S5 lost 90% of its original specific activity under acidic condition and about 50% at

Table 2. Activity of catechol 2,3-dioxygenase with different substrates

Substrate	Specific activity[a] (U g^{-1} protein)	Relative activity[b] (%)
catechol	13.4 ± 0.42	100.00
3-methylcatechol	1.8 ± 0.14	13.67
4-methylcatechol	14.2 ± 0.75	106.33
4-chlorocatechol	27.2 ± 0.04	203.80

[a]Catechol 2,3-dioxygenase activity was assayed spectrophotometrically at 375 nm. Data are expressed as mean ± standard deviation (n=3).
[b]Expressed as percentage of specific activity with catechol.

pH 9.0 (Fig. 2C). Catechol 2,3-dioxygenase isolated from another thermophilic strain *Bacillus thermoleovorans* A2 showed the highest activity at pH 7.2 (Milo *et al.*, 1999).

In order to calculate K_m and V_{max}, the activity of catechol 2,3-dioxygenase from *Planococcus* sp. S5 was measured at different substrate concentrations as detailed in materials and methods (Fig. 2D). The K_m and V_{max} values were 42.70 μM and 329.96 mU, respectively. Much lower K_m values of 4.0, 1.4 and 11 μM were observed for purified catechol 2,3-dioxygenase from *Pseudomonas* sp. G7, *Pseudomonas putida* mt-2 and *Pseudomonas* sp. ND6 (Cerdan *et al.*, 1995; Jiang *et al.*, 2004), while the K_m of catechol 2,3-dioxygenase from *Stenotrophomonas maltophilia* KB2 crude extract was two-fold higher than that of the enzyme from S5 strain (Wojcieszyńska *et al.,* 2011). During our studies on the kinetic properties of catechol 2,3-dioxygenase, enzyme inhibition was observed at > 120 μM substrate (Fig. 2D). Inhibition of catechol 2,3-dioxygenase at lower concentrations of catechol (above 2 μM) was described by Takeo *et al.* (2007) for the wild-type enzyme from an aniline-degrading strain *Acinetobacter* sp. YAA.

Since the aromatic ring fission by catechol 2,3-dioxygenases occurs *via* an electrophilic aromatic substitution mechanism (Bugg, 2003), to determine the substrate specificity of C23O from *Planococcus* sp. S5 substrates with either activating (3-methylcatechol, 4-methylcatechol) or deactivating (4-chlorocatechol) substituents were used. Additionally, Klečka & Gibson (1981) and Polissi & Harayama (1993) have observed that intermediates of chloro- and methylcatechols inactivate C23Os by chelating the divalent kations necessary for its activity. Moreover, inactivation of these enzymes can be caused by oxidation of the ferrous iron in the active site (Riegert *et al.*, 2001). The substrate specificity of C23O from *Planococcus* strain S5 with the catecholic compounds tested are shown in Table 2. The enzyme prefered catechols with a substituent in the *para*-position (4-chlorocatechol, 4-methylcatechol) over the *meta* position. It showed a remarkably high relative activity towards 4-chlorocatechol (203% of that with catechol). The activity towards 4-methylcatechol was comparable to that to catechol, while only marginal activity was detected with 3-methylcatechol (13.4% of that with catechol). The high activity towards 4-chlorocatechol distinguishes the dioxygenase studied from other C23Os (Kim *et al.*, 1997; Milo *et al.*, 1999).

Low preference for 3-methylcatechol was also observed for C23O from *S. solfataricus* (Chae *et al.*, 2007), while 2,3-dioxygenases from *P. putida* UCC2 (Wallis & Chapman, 1990) and *Rhodococcus* sp. strain DK17 (Kim *et al.*, 2005) cleaved 3-methylcatechol at a higher rate than catechol. Cho *et al.* (2010) have revealed that in substrate-bound methylcatechol 2,3-dioxygenase from strain DK17 3-methylcatechol interacts with the iron *via* a single hydroxyl group. The β-hairpin structure of the active C-domain of the enzyme forms part of the substrate binding pocket, responsible for the substrate specificity as well as its correct positioning in the active site. Based on the activities of C23O from strain S5 observed with *para*- and *meta*-substituted catechols as substrates, we assumed that the active site of the enzyme differed from that of C23O from strain DK17. Probably substituents in the *meta*-position comprise a steric hindrance for the correct orientation of the substrate in the active site of C23O from *Planococcus* sp. S5, resulting in low activity against 3-methylcatechol. Chloro- and methylcatechols are substrates that cause inactivation of catechol 2,3-dioxygenases (Bartels *et al.*, 1984; Takeo *et al.*, 2007). The high activity of C23O from *Planococcus* sp. S5 against 4-chlorocatechol and 4-methylcatechol, suggests the presence of a mechanism for reactivation of inactivated enzyme in strain S5. Reactivation of extradiol enzymes by the action of a protein similar to ferredoxin was described earlier by Polissi & Harayama (1993) and Hugo *et al.* (1998). However, such an assumption needs to be verified by a detailed analysis of the entire operon responsible for phenol degradation in strain S5.

Nucleotide sequence of catechol 2,3-dioxygenase gene

Genes encoding catechol 2,3-dioxygenases are located on plasmids (Cerdan *et al.*, 1995; Stilwell *et al.*, 1995; Yrjälä *et al.*, 1997; Jiang *et al.*, 2004) or, as in *Pseudomonas* sp. strain KL28 and *Comamonas testosteroni* TA441, on the chromosome (Arai *et al.*, 2000; Jeong *et al.*, 2003). Our previous studies indicate that strain S5 contains plasmid DNA (Łabużek *et al.*, 2003). Therefore we applied PCR to amplify catechol 2,3-dioxygenase-encoding gene from *Planococcus* sp. S5 using primers 2,3D_zewF and 2,3D_zewR and plasmid or chromosomal DNA as a template. A PCR product of about 900 bp was successfully obtained only with plasmid DNA as template indicating that the gene encoding catechol 2,3-dioxygenase in strain S5 is located on the plasmid. Sequencing of that PCR product resulted in a 917-nucleotide sequence (GenBank ID: HQ223337) which showed 100% homology with the sequence of the C23O gene from *Pseudomonas* sp. PD10, 99% homology with genes for C23Os from *Pseudomonas* CF600, *Pseudomonas* sp. PD7, or *P. aeruginosa* and 95% with the gene from *P. mendocina* PC. It is interesting to note that the extradiol dioxygenase from a Gram-positive strain shows such a high homology with enzymes from the Gram-negative *Pseudomonas* strains. A remarkable degree of homology of nucleotide and amino acid sequence of C23O from Gram-positive and Gram-negative strains was also indicated by Candidus *et al.* (1994) for *Rhodococcus rhodochrous* CTM. Such a high homology of nucleotide sequences indicates a very recent common genetic origin of all these enzymes and horizontal transfer of the gene coding for catechol 2,3-dioxygenase in many bacterial strains. Such gene transfer of *tdnC* encoding C23O from *Pseudomonas putida* UWC (pD10, pQKH6) to *P. fluorescens, P. chlororaphis, P. aureofaciens* and a *Bordetella*-like species has been reported by Hill *et al.* (1994). Horizontal transfer of the C23O gene was also observed by Wang *et al.* (2007) between endophytic and rhizosphere bacteria and by Jussila *et al.* (2007) between *Pseudomonas* and *Rhizobium*.

In summary, the Gram-positive *Planococcus* sp. strain S5 exhibited simultaneously catechol 1,2- and 2,3-dioxygenase activities when grown on low phenol concentra-

tions, while at higher initial phenol concentrations only the activity of C23O was detected. The extradiol enzyme from strain S5 degrading 4 mM phenol was optimally active at 60°C and pH 8.0. A remarkable preference of this dioxygenase for catechols with a substituent in the *para*-position, especially towards 4-chlorocatechol, distinguishes it from other C23Os from Gram-positive and Gram-negative bacteria. The properties of the described enzyme make it useful for bioremediation applications.

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